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MITSG 89-16
May 1989

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May 1989
NA 86AA-D-SG089
R/L 20

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RELATED RESEARCH

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Lee, Anne, and Langer, Robert. **SHARK CARTILAGE CONTAINS INHIBITORS OF TUMOUR ANGIOGENESIS.** MITSG 84-8J. 5pp. No charge.

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Lee, A. K. U., von Beuzekom, M., Glowacki, J., and Langer, R. **INHIBITORS, ENZYMES, AND GROWTH FACTORS FROM SHARK CARTILAGE.** MITSG 82-25. 4pp No charge.

1. ABSTRACT

Most solid tumors start as a small mass of avascular tissues which must be nourished by the host's vascular network in order to grow, and later, metastasize. Thus an inhibition of neovascularization, or anti-angiogenesis, is a possible therapeutic approach to controlling the growth of tumors. Cartilage, a source of this inhibitor, is avascular and rarely invaded by neoplastic tissues. Both veal and rabbit cartilages have demonstrated anti-angiogenic activities. However, the rate limiting factor in purifying and characterizing this substance has been the enormously large supply of cartilage required for extraction and isolation.

An abundant source of cartilage for anti-angiogenic factor was sought in sharks, because their skeletal tissue is entirely cartilage. When basking shark fin cartilage was extracted for 41 days in 1M guanidine solution and tested for anti-angiogenic activity against V2 carcinoma in the rabbit cornea, the vascular growth of the treated animals was 25% that of the control. In a comparable extract of veal scapular cartilage, only 60% inhibition was demonstrated. A higher level of anti-angiogenic activity was observed in the shark than in veal at this "crude extract" stage.

Histologically, shark fin cartilage was discovered to be composed of a tightly bound matrix. 41 days of extraction in 4M guanidine was not able to disrupt the collagen-proteoglycan matrix.

In addition, shark cartilage contains inhibitors of many proteases. Weak inhibitory activities were observed in the action of trypsin, plasmin and chymotrypsin. Also, shark cartilage contain inhibitors of collagenase specific for the type I and type IV collagens. Type I collagenase inhibitor, which was partially purified in a A-1.5M molecular sieve chromatography, has an approximate molecular weight of 35,000.

Besides these protease inhibitors, shark cartilage also contains lysozyme and cellular growth factor. In addition, an in vitro assay for angiogenesis inhibitors using capillary endothelial cells was developed.

2. INTRODUCTION

Cartilage is an avascular tissue which is resistant to invasion by neoplasms, and is a known source of collagenase inhibitor and anti-vascular factor. Our studies here explored the possibility of restricting tumor growth by inhibiting the vascularization of tumors with extracts from shark cartilage. The possibility that shark cartilage may contain many useful bioactive compounds is also explored. Finally, a new in vitro assay to screen anti-angiogenesis compounds was developed.

3. BACKGROUND

3.1 Anti-angiogenesis: An Approach to Controlling the Growth of Solid Tumors

Almost all solid tumors start as small aggregates of avascular malignant cells. At this stage, cells exchange nutrients and wastes by simple diffusion. However, as the tumor grows in volume, it becomes necessary for it to find an alternate method of sustaining itself, because by diffusion alone an adequate exchange of metabolites is not possible for tumors beyond a size of 2 mm in diameter. In a growing three dimensional tumor, the ratio of the surface area to the volume of a tumor decreases and imposes a limitation on the tumor size. Tumor cells solve this problem by secreting TAF, or tumor angiogenesis factor, which is mitogenic to the host's vascular endothelium and induces new capillaries to penetrate the tumor. This phenomenon is called "tumor angiogenesis". With the extablishment of a vascular network, the tumor cells can grow exponentially and display their full potential for growth and metastasis.

In 1971, Folkman proposed a new therapeutic approach based on the principle that if the capillary growth to the tumor is inhibited, the tumor must remain in an avascular, or dormant, state. He coined the term "anti-angiogenesis" to represent this concept.

There are four possible ways in which tumor angiogenesis can be prevented: (a) the synthesis of TAF can be blocked directly, (b) TAF can be inhibited while it is in transit by making specific anti-TAF or antibody, (c) endothelial cells, which make up the walls of the capillaries can be prevented from proliferating. Such an anti-mitogenic factor will obstruct the formation of building materials for capillaries, (d) lastly, one can look

at the inhibition of capillary penetration of tumor. An inhibitor which can obstruct capillary growth to a tumor will prevent tumor vascularization. To achieve anti-angiogenesis, an approach to blocking capillary advancement by preventing the degradation of the surrounding connective tissues will be explored.

3.2 Cartilage as a Source of Anti-angiogenic Factor and Protease Inhibitor

Cartilage is one of the few tissues in the body that is not invaded by blood vessels. At the embryonic stage cartilage is vascularized, but the blood vessels disappear in the early neonatal period. A factor inhibiting capillary proliferation may be activated at this stage. Cartilage, whose major constituent is collagen, is rarely invaded by neoplastic tissues. It seems possible then, that an anti-collagenase factor(s) may be a normal constituent of the tissue. Thus cartilage was speculated as a source of anti-collagenase, anti-vascular and anti-tumor substances.

Our laboratory has found that both veal and rabbit cartilage contain an inhibitor to tumor neovascularization (Langer, et al., 1976). The single factor most limiting to the further study of this substance is its supply. Cartilage is present only in small quantities in mammalian species. Therefore, shark, which is abundant and possesses an endoskeleton composed almost entirely of cartilage, is targeted as a potential source of an inhibitor of neovascularization.

4. MATERIALS

All reagents used in the experiments, unless otherwise indicated, were purchased from Baker Chemical Co., Bio-Rad, Fisher Scientific Co., Malinkrodt, Inc., Sigma Chemical Co. or Calbiochem. All reagents were prepared with either double glass distilled or distilled water.

All spectrophotometric measurements were taken with a Gilford spectrophotometer (Model 250) with a quartz cuvette. A Mettler electronic balance (Model H15) was used to weigh small samples and chemicals. In all cases, a MRBA lyophilizer (Model 10-148, serial #1029) was used to freeze dry samples. A Beckman centrifuge (Model TJ-6R. cat. #339235 serial #582) was used for low speed centrifuging. Measurements of pH were performed on a Beckman zero-matic pH meter with a sensorex combination electrode. A vortex-Genie (Fisher Scientific) was used for mixing. Pipetting was performed with automatic adjustable pipettes (p-20, p-200, p-1000, and p-5000 Pipetman, Rainin Instrument Co.)

In purifying collagen from rat tail tendon, a Sorvall refrigerated centrifuge (Model RC-5. Du pont. Serial #7500510) and Beckman ultracentrifuge (Model L5-75 cat. #338556, serial #470) were used. Radioactive collagen was counted on Beckman scintillation counter (LA-230 serial #0100085) filled with 5 ml of Hydrofluor (National Diagnostic) scintillation fluid.

Protein separation was done using the following equipment: A Gilson fraction collector (Model MFK, serial #168E0081) and Eldex Universal Fraction Collector (V-1A) were used to collect samples from an A1.5M agarose column. A Microperpex pump (LKB. cat. #2132.001) was attached to the column (Pharmacia). A SDS PAGE vertical slab apparatus (Bio-Rad Model 220) was used to check for the purity of sample. Standard proteins of known molecular weight (Bio-Rad) were: Bovine serum albumin (m.w. 68,000), ovalbumin (m.w. 45,000), carbonic anhydrase (m.w. 30,000), soybean trypsin inhibitor (m.w. 21,000) and lysozyme (m.w. 14,300).

Basking shark fin and vertebrae were bought from Fresh Water Co. (Boston, Mass.) and dogfish sharks were caught fresh 30 miles off Nahant (Mass.) in a commercial fishing boat with the assistance of Maggie Linskey of MIT Sea Grant. The basking shark was approximately 20 feet in length. Connective tissue on the basking shark cartilage was scraped off and fins and two parts of the vertebrae were stored separately at -20 °C for 2 and 4 months respectively before extraction. The vertebrae consists of a central circular concave portion, the centrum, or body, and a neural arch which is dorsal to the centrum. Dogfish shark was approximately 3 feet in length. Upon catching the shark by gillnets, fins were immediately removed and the connective tissues were scraped off using a scalpel (blade 10). Extraction in ice-cooled guanidine HCl solution started less than 2 hours after the catch.

5. METHODS

5.1 Cartilage Extraction Procedure

Basking shark cartilage which had been frozen at -20 °C was thawed, chopped in 1 cm pieces and extracted with different dissociating agents for different durations. Basking shark fin, centrum and neural arch of the vertebrae were extracted in 1M guanidine HCl in .02M MES buffer at pH 6.0 for 24 hours in large quantities by Monsanto Co. (St. Louis, MO). Basking shark fins were extracted only in small quantities (5-20 grams) in our laboratory due to the large amount of space required for the extraction procedure. All dissociating chemicals were prepared with .02M MES buffer and pH was adjusted to 6.0 using reagent grade NaOH or HCl. At room

temperature (25 °C), shark fins were extracted in .5M, 1M, 2M, 4M, 6M Urea for 1, 2, 3 days. In addition, shark fin cartilage was incubated in 1M guanidine for 41 days and 6 months. Dogfish shark fin cartilage pieces were extracted only in 1M guanidine HCl solution at 25 °C for 1, 3, 14 and 21 days. Using sterile glass distilled water, the cartilage was extracted at 1:10 (w/v) ratio using a liter chromatography jar as a vessel. Mild agitation was applied with an overhead mechanical stirrer with a metal blade (30 RPM).

After extraction, the cartilage pieces were removed and the solution was exhaustively dialyzed in sterile double distilled water at 4 °C using 3500 m.w. cutoff dialyzer tubing (Thomas cat. #3787-H47) to remove salts. Over a week period, 100 ml of extracted solution was dialyzed against 4 liters of water which was changed five times. As the salt was removed, extracted material reaggregated. Thus, at the end of dialysis, the solution was both centrifuged and filtered through Whatman 1 (Fisher. cat. #09-8055) to remove all solid matter. Centrifugation was done simply to speed up the filtering process, because the precipitate was of such a texture as to quickly clog the filter paper. However, centrifuging at 10,000 g for 20 minutes does not remove all matter, so both centrifuging and filtering were done. The filtrate was lyophilized. The lyophilized material is called the "crude extract".

5.2 Assay for Anti-angiogenic Activity

Rabbit Cornea Assay

Anti-vascular activity was quantitated by a rabbit cornea assay. Slow-release polymer pellets which contained extracts of basking shark fin cartilage were implanted in the pocket of rabbit corneas adjacent to V2 carcinoma (Shope virus-induced papilloma) and the length of the maximum blood vessel was measured. Fresh dogfish cartilage was also tested for inhibition of blood vessel growth. Only a few samples could be tested, because of the cost and the difficulty of the assay. For more details in these methods see (Lee and Langer, 1983).

Slow Release Polymer

Slow release polymer pellets were prepared in two ways: aqueous dispersion and carrier methods. In the aqueous dispersion method, 10% ethylene-vinyl acetate copolymer (EVA, Du pont Chemical Co., Wilmington, Delaware. Product name, Elvax 40) in methylene chloride (Fisher Scientific. cat. #D-123) is vortexed with a sample which is pre-mixed with water. Various crude extracts as well as 1 and 41 day crude extracts of

basking shark fin from 1M guanidine solution were made by this method. 1 day extract and 41 day extract pellets implanted in the rabbit cornea contained approximately .11 mg of crude extract.

In the carrier method, the active substance was incorporated in polymer pellets along with carrier protein (Rabbit serum albumin; Sigma). Only collagenase inhibitor was delivered by this method. 6.2 mg of partially purified collagenase inhibitor from basking shark cartilage (protein by weight. (Lowry)) was incorporated in 10% EVA with 16 mg of rabbit serum albumin (Sigma). Each polymer pellet implanted in the rabbit cornea contained approximately .17 mg of partially purified collagenase inhibitor.

5.3 Assays for Other Bioactive Compounds in Shark Cartilage

Agar Assay for Trypsin, Chymotrypsin and Plasmin Inhibition

Introduction

Trypsin, chymotrypsin and plasmin inhibitory activities were measured using a Bio-Rad protease detection kit (Bio-Rad cat. #500-0010, 500-0003). The Bio-Rad kit consists of casein substrate agar tablets, plasminogen and urokinase.

Bio-Rad's substrate gel tablets provide a rapid, convenient method for preparing agar diffusion plates for use in the detection of protease activities such as trypsin and plasmin. Bovine pancreatic trypsin treated with TPCK (Worthington) to inhibit contaminant chymotrypsin activity was used. The tablets produce a 1% agar gel containing a bovine casein preparation in a .05M Tris solution buffered to .15M NaCl solution at pH 7.2. Protease diffusion into the substrate gel is accompanied by digestion of the casein. This causes a transparent ring to be formed around the sample wells in a turbid gel. The diameter of the ring is a measure of the proteolytic activity of the sample.

Plasminogen activation

Plasminogen is in inactive form and must be activated by urokinase. A urokinase sample was dissolved in 0.01M K_2HPO_4 at pH 7.25, containing 0.15M NaCl and 1 mg bovine serum albumin/ml to give a solution containing 300 to 500 Ploug units/ml. A Ploug unit is an arbitrary unit defined from a crude preparation of urokinase. Approximately, 5-8 units of urokinase activity exists per ml of normal male urine.

Equal volumes of urokinase solution and serum containing plasminogen were combined and allowed to stand for 30 minutes at 25 °C to activate. This solution was used to detect plasmin inhibitory activity.

Experimental procedure

Two substrate gel tablets were placed in an 18 mm x 150 mm test tube and 10 ml of distilled water was added and allowed to hydrate for 15 minutes at room temperature. The mixture was vortexed thoroughly to obtain a complete suspension of the tablets. Failure to adequately solubilize the substrate (casein) and buffer components before heating resulted in a heterogenous plate.

The suspension was heated in a boiling water bath for 3-5 minutes until the agar dissolved completely. It was vortexed and visually checked to verify that all solid had completely dissolved. Care was taken not to overheat the sample, as this resulted in precipitation of casein. Hot agar melt was pipetted onto a plastic diffusion plate and the solution was cooled to room temperature. When the gel cooled, 4.0 mm diameter sample wells were punched in the gel.

10 µl samples were pipetted into each well. Shark crude extract was dissolved in .05M Tris containing .15M NaCl, pH 7.2 and preincubated for 30 minutes in solution at 37 °C in previously prepared protease solution, plasmin, chymotrypsin and trypsin, in equal volumes. The plate was incubated at 25 °C for 22 hours. Controls were Tris buffer and the appropriate protease (plasmin, chymotrypsin or trypsin) for each experiment.

Termination of the protease digestion was accomplished by overlaying the plate with a solution of 3% (v/v) acetic acid. After 10 minutes the plate could be rinsed with water and the diameter of the rings measured.

Lysozyme assay

Lysozyme activity was assayed according to the method of Osserman (3).

Growth factor

The growth factor activity from shark cartilage crude extract was partially purified on a molecular sieve chromatography and assayed for the growth factor 3T3 cells according to the method of Klagsbrun (4).

5.4 In Vitro Capillary Cell Angiogenesis Inhibitor Assay

Bovine capillary endothelial cells (BCE), a gift from Dr. Judah Folkman and C. Butterfield, were isolated and maintained in culture in Dulbecco's modified Eagles medium with 10% calf serum (DMEM/10). The cultures were supplemented with retina-derived growth fator (5 µl/ml).

Inhibition Assay

BCE were plated (10^4 cells/ 0.5 ml) in DMEM/5 into gelatin coated 24 well tissue culture dishes (Nunc, Denmark; Vanguard International, New Jersey) (0.5 ml of 1.5% Difco gelatin in PBS per well), and were allowed to attach overnight. The following day, the unattached cells were removed and the attached cells refed with DMEM/5. The test substances and RDGF (10 µl/ml) were added simultaneously in volumes that did note exceed 10% of the final volume. Seventy-two hours later the medium was aspirated, the cells were washed with 0.5 ml of PBS, removed by trypsinization (0.5 ml 0.05% trypsin-0.02% EDTA) and counted electronically with a Coulter Counter.

Percent inhibition was defined as:

$$100 - \left\{ \frac{\text{BCE exposed to stimulus and test sample - control BCE}}{\text{BCE exposed to stimulus - Control BCE}} \times 100 \right\}$$

Reversibility

To assess if the observed effects were due to inhibition and not due to toxicity, the reversibility of the inhibition was examined. The methods for the inhibition assay described above were followed. On day 5, cells from representative wells of each condition: cells with no additions, cells with added growth factor, and cells with added growth factor and test sample were counted. The remaining cells were washed twice with 0.5 ml of PBS to remove added factors, and either refed with DMEM/5 alone or DMEM/5 plus growth factor. These cells were counted 3 days later. Results of this study would reveal reversibility if cells that had been exposed to inhibitor through day 5 could respond to readded growth factor by dividing at the same rate as control cells exposed to growth factor.

Factor Preparation

Growth factors

Extracts of bovine retinas (retina-derived growth factor; RDGF), which contain predominantly Class I heparin binding growth factors (HBGFI) or acidic fibroblast growth factor (aFGF) were prepared as follows. Retinas were removed from bovine eyes and incubated in Hank's balanced salt solution (1 retina/ml) for 3 hours at room temperature, then removed

by centrifugation at 3000 RPM for 10 min. Partial purification was accomplished by an acid precipitation step. The supernatant was adjusted to pH 4.0 M using 1.0 M HCl and the resultant precipitate removed by Centrifugation at 10,000 RPM for 30 min. The supernatant was readjusted to pH 7.4 with 1.0 M NaOH, filter-sterilized (0.2u, Millipore filter) and stored at -20 °C.

Vitreous-derived inhibitor

Bovine vitreous-derived inhibitor previously shown to be anti-angiogenic, was obtained from G. Lutty (Wilmer Opthalmological Institute, Johns Hopkins Hospital, Baltimore, MD).

6. RESULTS

6.1 Shark Cartilage Extraction

The approximate weights of the crude extract obtained from 10 grams of cartilage extraction under various conditions are reported (Table 1). About 0.2% (by weight) of the basking shark fin cartilage was obtained as a crude extract when extracted in 0.5M, 1M, 2M guanidine for 24 hours and 48 hours and .25% (by weight) when extracted in 3M and 4M guanidine. A larger mass of crude extract, 0.46% (by weight) of the cartilage was released with a prolonged incubation of 180 days in 1M guanidine. In the extracts using lower concentrations of urea and $MgCl_2$ at 24 and 48 hours of extraction time, approximately 0.15 to 0.2% (by weight) of the basking shark fin cartilage was obtained as a crude extract.

In the basking shark vertebrae, the centrum and the neural arch, which were extracted for 24 hours in 1M guanidine released crude extract mass which amounted to 0.6 and 0.4% by weight of the cartilage, respectively.

Dogfish shark fin extracted in 1M guanidine for 15 days released about 0.2% (by weight) of cartilage as crude extract.

6.2 Anti-angiogenesis Activity

Crude extracts from basking shark fin cartilage extracted in 1M guanidine for 41 days, proved to contain a strong anti-angiogenic activities when tested in the rabbit cornea assay. Weaker activities were noted in the crude extracts obtained from 1M guanidine extraction of veal scapular and basking shark fin for 1 day. The activities were plotted and compared (figs. 1-4). Also, a list of shark samples tested in the rabbit cornea which did not contain any anti-angiogenic activity or caused inflammation is compiled (table 2).

The most significant inhibition of tumor neovascularization was obtained with the shark cartilage fin extract in 1M guanidine for 41 days (0.11 mg by weight per polymer pellet). Three different tests were conducted, and, in every case inhibition was observed. The results show that 19 days after implantation all controls had large three dimensional tumors with an average maximum vessel length of 6 mm which covered half of the rabbit's cornea (Fig. 2a). In contrast, none of the treated corneas had 3 dimensional tumors. All showed sparse vascularization with clear zones of inhibition around the slow release polymers containing shark extract (fig. 2b)). The average maximal vessel length was 1.5 mm, 25% of the controls.

Crude extract obtained from a 1M guanidine extraction of veal scapular cartilage for 1 day demonstrated some inhibition of tumor neovascularization in the three animals tested (fig. 3). However, the inhibitory activity was not as great as that of crude extract obtained from 1M guanidine extraction for 41 days. The average maximal vessel length was 4 mm, 60% of the controls.

In many other guanidine extracted fractions and fresh samples tested, either inflammation or rampant capillary proliferation (and thus a three dimensional tumor) were observed. Although the crude extract from the basking shark fin cartilage extracted for 41 days in 1M guanidine caused a significant inhibition of tumor neovascularization (fig. 1), 2M guanidine, 41 days extracted crude extract caused severe inflammation (Table 3). The rabbits had to be sacrificed on day 10. Thus, it seems possible that different substances are released from the cartilage at different molar concentrations of guanidine.

Crude extract obtained from a 1M guanidine extraction for 1 day of basking shark neural arch and the centrum (0.15 mg per pellet) did not inhibit capillary growth (Table 2). Inhibitory activities from the fresh cartilage of the dogfish shark (1 mm^3 of cartilage per cornea) were also insignificant (Table 2). Fresh dogfish shark cartilage tested included the fin (obtained from an animal killed three hours before the samples were taken) and the cartilage from the baby shark, invariably in the bellies of female dogfish sharks. Egg white lysozyme, which was included as a control, did not demonstrate any inhibition (Table 2).

6.3 Other Activities

Anti-protease activity

Trypsin inhibitory activity

Weak trypsin inhibitory activities were found in basking shark fin cartilage extracted in guanidine, urea, $MgCl_2$ for different durations and

concentrations. Only low levels, between 10-20%, of trypsin inhibitory activities were observed in all crude extracts tested (Table 3).

A more quantitative determination of trypsin inhibition was made with the TAME assay. In the TAME assay, percent inhibition was calculated as the (slope of control - slope of sample) (100)/(slope of control) (Table 4). It is important to note that when 0.5 mg of both 1M guanidine extracts of shark fin and veal scapular cartilage were tested against 1 μg of trypsin, the veal exhibited three times more inhibitory activity.

Plasmin and chymotrypsin inhibitory activities

Between 20-30% inhibition of 15 mg of chymotrypsin (as compared with the control) was observed with 150 mg of crude extract from basking shark extracted for 41 days in 1M guanidine (Table 5). No chymotrypsin inhibition was found in the 1 day extract of fin. The results seem to indicate that the fin must be extracted over a period of time to release enough chymotrypsin inhibitor to be detected in the casein agar assay, although the neural arch and the centrum seemed to release enough in one day in 1M guanidine to exhibit 30% inhibition.

A similar amount of plasmin inhibition (between 27-40%) of six Ploug units of plasmin was found in all of the four samples mentioned above (Table 5).

Anti-Collagenase Activity

Inhibition of collagenase for type I collagen

The collagenase harvested from rabbit cornea culture of different batches exhibited varying amounts of collagenase activity (0.05, and 0.08 units). Collagenase was activated with trypsin. The standard curves for each collagenase inhibitor (in the crude extracts obtained from each extraction conditions) could not be constructed because of the limited amount of the material. Since, without the standard curve, the linearity of the inhibitor concentration with respect to collagenase inhibitory activity can not be assumed, samples were separated according to the activity of collagenase used and analyzed individually.

The activated collagenase from every batch was preincubated with a specified amount of crude extract described in tables 7, 8, and 9 for 30 minutes at 37 °C enabling collagenase in the media to interact with the collagenase inhibitor in the crude extract. This solution is called the inhibitor solution. 200 μl of the inhibitor solution was added to the tissue culture well containing 50 μg of ^{14}C-labelled type I collagen substrate and incubated at 37 °C for 2.5 hours. Collagen fibrils cleaved by the collagenase rose up to the supernatant and were counted in a scintillation counter. The

counts of the samples were compared as a percentage of the control (collagenase alone) to determine their inhibitory activity.

When 0.05 units of collagenase was incubated with the crude extract concentrations of 30, 15, 7.5, and 3.75 mg per ml of media, a similar amount of activity was observed in basking shark fin and veal scapular cartilage derived crude extracts (Table 6). Both crude extracts seemed to inhibit the collagenase in the media to a saturation point (90% inhibition) at the crude extract concentration of 15 mg/ml.

When a higher concentration (0.08 units) of collagenase in the media was used to test for collagenase inhibitory activity, results consistant with those in the table 7 were found. Shark neural arch exhibited a lower collagenase inhibitory activity than that of veal (Table 7). Basking shark centrum exhibited strong collagenase inhibition, stronger than that of neural arch but weaker than that of veal extract when four concentrations (30, 20, 10, 5 mg/ml) of crude extracts were compared. Although bovine serum albumin is known as a "sticky" protein (one which loosely binds to most enzymes) almost no collagenase inhibitory activity (17% at 30 mg/ml) was seen when bovine serum albumin was included as a control.

Inhibition of collagenase for type IV collagen

Only a preliminary assay for the inhibition of the collagenase for type IV collagen was performed, because a quantitative measurement would require a purer sample of collagenase which was not available.

The crude extracts were tested for collagenase inhibition by using collagenase specific for type IV collagen from a cell culture of a transformed human skin fibroblast (Hut-11). To prevent any trypsin and chymotrypsin from attacking type IV collagen from the hamster lung basement membrane, soybean trypsin inhibitor and TPCK, a chymotrypsin inhibitor, were added to the media. 50 µl of media was able to degrade 0.2µg of type IV collagen per minute, i.e. the specific activity of collagenase was 0.2 unit. All the crude extracts were obtained by extraction in 1M guanidine.

The basking shark fin cartilage obtained from 1 day extraction showed the highest amount of collagenase inhibition (Table 8). The 180 day extraction of the same cartilage did not show any collagenase inhibitory activity. Possibly a prolonged exposure to guanidine or incubation at 25 °C may have denatured the compound responsible for the type IV collagenase inhibition. Cartilage extracts from different parts of the shark skeleton, displayed different degrees of collagenase inhibition in the decreasing order of fin, neural arch and centrum (Table 8). Veal scapular cartilage extract exhibited as much inhibition as the extract from

basking shark neural arch, but did not exceed that of the fin. An insignificant amount of inhibition was observed in the dogfish extracts.

Growth factor

To purify cellular growth factor, A-0.5M molecular sieve chromatography (exclusion limit 0.5 million daltons) was used to separate the shark crude extract (1M guanidine extracted for 1 day) by molecular weight. The eluant was collected, and lyophilized. The material was resuspended in 50 µl of water and tested for growth factor activity by measuring the incorporation of radioactivity labelled thymidine by 3T3 cells.

The background count of 7000 cpm was substrated from the count of the experimental samples before plotting in a bar graph (fig 6). The bars represent the amount of radioactivity incorporated by the cells incubated with shark crude extract samples from the column.

Growth factor activity was seen in a broad molecular range, because of the limitation inherent to the column. However, most of the growth factor activity was found in between the molecular weight of 25,000 and 5800 and the activity peaked between 17,800 and 14,300 daltons.

Lysozyme activity

Shark lysozyme in the crude extracts (µg lysozyme/mg crude extract) was quantitated from the standard lysozyme curve. The lysozyme standard curve was constructed by the measurements derived from the lysis of micrococcus lysodeikticus by egg white lysozyme. The value plotted on a semi-logarithmic paper was the diameter of micrococcus lysodeikticus lysis.

Lysozyme activity was found in all the 24 hour extracts of 1M guanidine of the basking shark fin and in the vertebrae (Table 9). Basking shark fin contained the least amount of lysozyme. Among the vertebrae parts, the centrum seemed to show a higher content of lysozyme (133 mg) than the neural arch (85 mg) when one mg of each crude extract was compared.

6.4 In Vitro Bioassays

Cartilage Derived Inhibitors

When the inhibitor was added to the BCE in the presence of RDGF, inhibition of cell growth was observed. The effect was dose-dependent;

22.5 ug/ml produced 18% inhibition; 45 ug/ml produced 49% inhibition and 90 ug/ml resulted in nearly total inhibition of BCE proliferation. Likewise, the peak fraction of the cartilage inhibitor inhibited the proliferation of BCE that had been stimulated by RDGF. Again, the effect was dose dependent with 25 ug/ml causing 34% inhibition and 50 ug/ml resulting in 60% inhibition and 100 ug/ml totally inhibiting BCE proliferation. When cartilage inhibitor was added to the BCE, either in the presence of purified basic FGF or purified brain-derived acidic FGF significant inhibition of proliferation was also observed.

Other Anti-angiogenesis Factors

An extract of vitreous has been demonstrated by Lutty and co-workers to inhibit RDGF-induced neovascularization in the CAM. When 30.8 ug/ml of the vitreous extract, concentrations similar to those used for the cartilage inhibitors, was added to the BCE in the presence of RDGF, 79% inhibition was observed.

We also assessed the effect of PF4 on BCE proliferation. PF4 has been shown to be an angiogenesis inhibitor, producing avascular zones in the CAM assay. When 400 ng/ml of PF4 was added to the BCE in the presence of RDGF, 24% inhibition was observed; 600 ng/ml inhibited 48%, 800 ng/ml inibited 52%, and 1 ug/ml caused 73% inhibition. PF4 at 2 ug/ml ws cytotoxic.

Controls

To determine if the observed inhibition was specific for anti-angiogenic substances, two other enzyme inhibitors (trypsin ovoinhibitor and pancreatic inhibitor), a collagenase inhibitor, (alpha-2 macroglobulin), and a glycosaminoglycan found in cartilage (chondroitin sulgate, type A), were assayed for their effect on the proliferation of BCE. All of these compounds were examined at 50 µg/ml, the dose of the cartilege inhibitor observed to produce maximal inhibition. Fifty ug/ml of cartilage inhibitor caused 91% inhibition of RDGF-induced proliferation. In contrast, the control substances at the same concentration did not have a significant effect on BCE proliferation. These results indicate that the inhibition mediated by anti-angiogenic factors is specific for those factors and is not caused by other enzyme inhibitors or cartilage-derived substances.

Reversibility

The goal of the reversibility studies was to determine if BCE could resue their normal growth rate following the removal of the inhibitors.

Thus, the slopes of the growth curves of treated cells were compared to the slope of the growth curves of untreated cells. Control cells (that had been fed DMEM/5 media on day 2 and 5) grew at a rate of 11,100 cells/day between days 5 and 8. Two inhibitor-treated cell populations were studied. In the first, cartilage inhibitor was added to cells along with RDGF, resulting in 77% inhibition orver the three day time course. On day 5, the inhibitor was removed and replaced with DMEM/5. The growth rate of those cells was 13,900/day in the subsequent 3 days (day 5 through day 8), which compares favorably with the untreated controls. In the second group, the cells received inhibitor and RDGF on day 2 and on day 5 the inhibitor was removed and replaced with DMEM/5 containing RDGF. The growth rate in this second group was 28,400 cells/day. The control cells for this group, which received DMEM/5 on day 2 and RDGF on day 5 grew at a rate of 24,800 cells/day between days 5 and 8. Thus, BCE that had been inhibited by inhibitor were able to grow at the same rate as untreated cells both in the presence and absence of RDGF, indicating that cytotoxicity is not the cause of the observed inhibition. All these results indicate the usefulness and specificity of this endothelial cell bioassay for studying and purifying angiogenesis inhibitors. Future studies will be aimed at using this assay as an initial screen for purified shark cartilage fractions, of which there will be many. Those that test positive will be examined in the more time consuming and expensive in vivo asssays - the chick CAM and rabbit cornea. These studies should lead to the eventual identity of the shark cartilage inhibitor.

7. ACKNOWLEDGEMENTS

This work, project # RL-20, was funded (in part) by the MIT Sea Grant College Program under federal grant #NA 86 AA-D-SG089 from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce.

8. REFERENCES

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Table 1: Crude Extracts[a] from Cartilage Extracted with Guanidine, Urea and $MgCl_2$

Cartilage Source	Dissociating Agent	Extraction[b] Time(Days)	Crude Extract[c] (mg)
Basking Shark Fin	.5M guanidine HCl	1	20
Basking Shark Fin	.5M guanidine HCl	2	20
Basking Shark Fin	1M Gu HCl(Monsanto)	1	20
Basking Shark Fin	1M guanidine HCl	2	20
Basking Shark Fin	1M guanidine HCl	180	46
Basking Shark Fin	2M guanidine HCl	2	20
Basking Shark Fin	3M guanidine HCl	1	25
Basking Shark Fin	3M guanidine HCl	2	25
Basking Shark Fin	4M guanidine HCl	1	25
Basking Shark Fin	.5M urea	2	15
Basking Shark Fin	1M urea	2	15
Basking Shark Fin	2M urea	2	20
Basking Shark Fin	4M urea	2	20
Basking Shark Fin	6M urea	2	25
Basking Shark Fin	1M $MgCl_2$	1	15
Basking Shark Fin	2M $MgCl_2$	1	15
Basking Shark Neural Arch	1M guanidine HCl (Monsanto)	1	40
Basking Shark Centrum	1M guanidine HCl (Monsanto)	1	60
Veal Scapular Cartilage	1M guanidine HCl (Monsanto)	1	80
Dogfish Fin	1M guanidine HCl	15	20

a. Crude extract is the lyophilized material obtained after the cartilage has been extracted, and the dissociating agents have been dialyzed.

b. Cartilage was extracted at 25°C.

c. 10 grams of cartilage was extracted in each case to obtain the reported amount of crude extract.

Table 2: Samples Which Were Either Inflammatory or Not Exhibiting Anti-angiogenic Activity in the Rabbit Cornea Assay

Cartilage Sample (no. of animals)	Cartilage or Polymer Implant	Appearance of Cornea 15 days After Implant
Basking shark fin extracted in 2M guanidine for 41 days (n=4)	.16 mg*	Inflammation[b]
Basking shark[c] neural arch (n=3)	.15 mg*	Three dimensional
Basking shark[c] centrum (n=3)	.15 mg*	Three dimensional
Fresh dogfish[a] shark fin (n=6)	1 mm^3**	Three dimensional
Fresh baby dogfish[a] shark fin (n=3)	1 mm^3**	Three dimensional
Egg white lysozyme (n=8)	.18 mg*	Three dimensional

a. Dogfish sharks were killed two hours before implant.

b. Inflammation was observed starting day 3 after implant. The animals were sacrified on day 10 due to severe inflammation.

c. Basking shark vertebrae were extracted in 1M guanidine for 1 day.

* Crude extract was implanted in polymer pellet made by the aqueous dispersion method (see procedure).

** Cartilage pieces were implanted.

Table 3: Inhibition of Trypsin by Various Basking Shark Cartilage Extracts

Dissociating Agent	Extraction (Days)	Concentration of the Solution 1M	2M	3M	4M
$MgCl_2$	1	+	+	+	
Guanidine HCl	1	+	+	+	+
Guanidine HCl	41	+	+	+	
Guanidine HCl	180	+			
Urea	2	+	+		+

Basking shark cartilage extracts were screened for trypsin inhibitory activity in 1% agar gel containing bovine casein. In sample wells, 10 µl of sample containing 50 µg of shark extracts were incubated with 10 µg of trypsin for 30 minutes at 37°C before placing the sample in the well. Diameter of casein lysis was measured. Inhibition was measured as a percentage of the uninhibited control containing only typsin. - represents trypsin inhibition less than 10%, + represents trypsin inhibition between 10-20%.

Table 4: Inhibition of Trypsin by Shark and Veal Cartilage Crude Extracts[a] using TAME[b] as Substrate

Sample	% Trypsin Inhibition
Shark	14
Veal	44

a. Veal scapular and shark fin cartilages were extracted in 1M guanidine HCl in .02M MES at pH 6.0 for 24 hours.

b. Tosyl-L-arginine methyl ester.

2.5ml of Tris buffer (.05M Tris, .0115M $CaCl_2$ at pH 8.0), .1 ml of inhibitor solution (5 mg/ml) and .1 ml of trypsin were incubated at 25°C for 30 minutes. The control consisted of 2.6 ml of Tris buffer, and .1 ml of trypsin solution. The trypsin inhibitory activity was assayed by mixing the enzyme-inhibitor solution or the control with .3 ml of stock TAME solution. The OD/min was graphed at 247 nm. The slope of the control was compared with the slopes of the samples containing the inhibitors to determine their trypsin inhibitory activities. Percent inhibition was calculated as the (slope of control/slope of sample)(100)/slope of control.

Table 5: Inhibition of Plasmin and Chymotrypsin by 1M Guanidine Extracts of Basking Shark Cartilage

Sample	Extraction (Days)	Chymotrypsin (%)	Plasmin (%)
Fin	1	0	27
Fin	41	22	40
Neural Arch	1	30	40
Centrum	1	30	40

10 µl of sample containing 150 µg of shark crude extracts were preincubated with either 15 µg of chymotrypsin or about 6 Ploug units of plasmin for 30 minutes at 37°C before being placed in the agar well. The diameter of casein lysis was measured. Sample inhibition was measured as a percentage of the uninhibited controls containing only enzyme.

Table 6: Inhibition of Collagenase (.05 units)[a] for Type I Collagen[b] by Veal Scapular and Basking Shark Cartilage Crude Extracts[c]

Sample	Concentration[d] (mg/ml)	% Inhibition[e]
Basking Shark fin (Monsanto)	30	93
	15	89
	7.5	70
	3.75	44
Basking Shark Neural Arch[f] (Monsanto)	30	90
	15	76
	7.5	6
	3.75	0
Veal Scapular (Monsanto)	30	100
	15	87
	7.5	72
	3.75	44

a. Collagenase was derived from rabbit cornea. Its specific activity was .05 unit . (see appendix 1 for calculation)

b. Type I collagen was purified from a rat tail tendon collagen.

c. Crude extracts were obtained by extracting cartilage in 1M guanidine for 24 hours at 25°C.

d. Concentration is expressed in mg of crude extract per ml of collagenase media.

e. % inhibition calculation was done by the method described in Appendix A-II.

f. See materials for definition.

Table 7: Inhibition of Collagenase (.08 units)[a] for Type I Collagen[b] by Veal Scapular and Basking Shark Cartilage Crude Extracts[c]

Sample	Concentration (mg/ml)[d]	% Inhibition[e]
Basking Shark[f] Neural Arch (Monsanto)	30	70
	20	37
	10	0
	5	0
Basking Shark[f] Centrum (Monsanto)	30	91
	20	49
	10	25
	5	23
Veal Scapular (Monsanto)	30	96
	20	92
	10	68
	5	41
Bovine Serum Albumin	30	17
	20	3
	10	0
	5	0

a. Collagenase was derived from rabbit cornea. Its specific activity was .08 unit (See Appendix A-I for calculation).

b. Type I collagen was purified from a rat tail tendon collagen.

c. Crude extracts were obtained by extracting cartilage in 1M guanidine for 24 hours at 25°C.

d. Concentration is expressed in mg of crude extract per ml of collagenase media.

e. % inhibition calculation was done by the method described in Appendix II.

f. See materials for definition.

Table 8 Type IV Collagen Specific Collagenase[a] Inhibitory Activity by Cartilage Extracted For Different Durations with 1M Guanidine[b]

Cartilage Source	Extraction Time (Days)	Collagenase[c] Inhibition
Basking Shark Fin	1	+++
Basking Shark Neural Arch	1	++
Basking Shark Centrum	1	+
Veal Scapular Cartilage	1	++
Dogfish Fin	15	+
Dogfish Fin	26	-
Basking Shark Fin	180	-

a. 50 µl of collagenase media was capable of breaking down 60% of total count of 2 x 10^4 cpm, or expressed in another way, contained .2 units of activity

b. Guanidine HCl in .02M MES at pH 6.0

c. 100 µg of various extracts were incubated with 50 µl of collagenase media from tansformed human skin fibroblast cells (Hut-11) for 10 minutes at 35°C before doing the enzyme assay.
+++ 100-71%, ++ 70-31%, + 30-21%, ± 20-10%,
- no inhibition.

Table 9. Lysozyme Activity in Basking Shark Cartilage Crude Extracts

Sample	Diameter of Lysis (mm)	$\frac{\mu g \text{ Lysozyme}}{mg \text{ crude extract}}$
Basking Shark Fin	9.3	61
Basking Shark Neural Arch	10.0	85
Basking Shark Centrum	11.8	133

Lysozyme activity was quantitated according to the lysis of *micrococcus lysodeikticus* in 1% agar suspension. The assay was run at 25°C for 24 hours. 15 µl of samples containing 75 µg of samples were applied in all cases and the diameter of the lysis was measured according to the metnod described in the procedure.

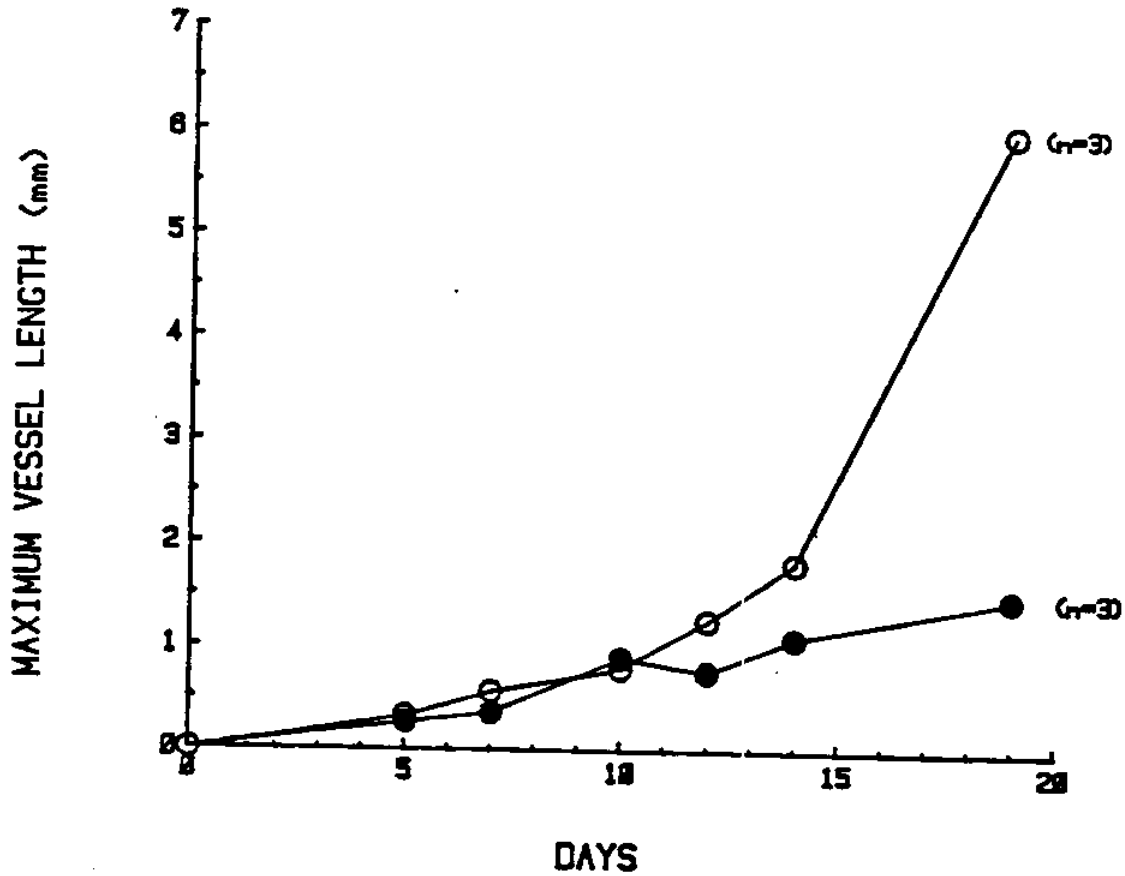

FIGURE 1: Inhibition of tumor neovascularization by basking shark fin cartilage extracted for 41 days in 1M guanidine.

The right cornea was implanted with a polymer pellet containing .11 mg of crude extract by weight (●). The left cornea was implanted with an empty polymer and served as a control (o). Rabbits were sacrificed on day 19, because the tumors in the left eyes became three dimensional and necrotic. The standard deviation of the maximal vessel lengths measured for the three animals tested with shark crude extract polymer pellets or empty pellets were as follows: for shark extract, on days 5,7,10,12,14,19 the standard deviations were .38, .47, .26, .29, .5, .3 respectively. For the controls, on days 5,7,10,12,14,19 the standard deviations were .2, .35, .44, .42, .67, 0 respectively.

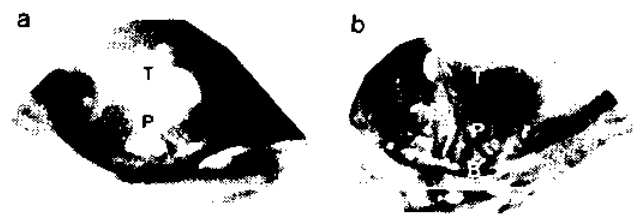

Fig. 2. Lower halves of rabbit corneas 19 days after the implantation of V2 tumor (*T*) and a polymer pellet (*P*) containing the inhibitor (a) or V2 tumor and a pellet without the inhibitor (b). The tip of the tumor was initially placed 2.0 mm from the edge of the cornea, and the pellet (surface area, 1 mm^2) was placed directly below it with its tip 1.0 mm from the corneal edge. The blood vessels appear as a black sheet sweeping over the polymer pellet and the tumor in the control (b). However, they do not grow nearly as rapidly in the experimental cornea (a) and form a zone of inhibition around the pellet. *B*, bottom of cornea.

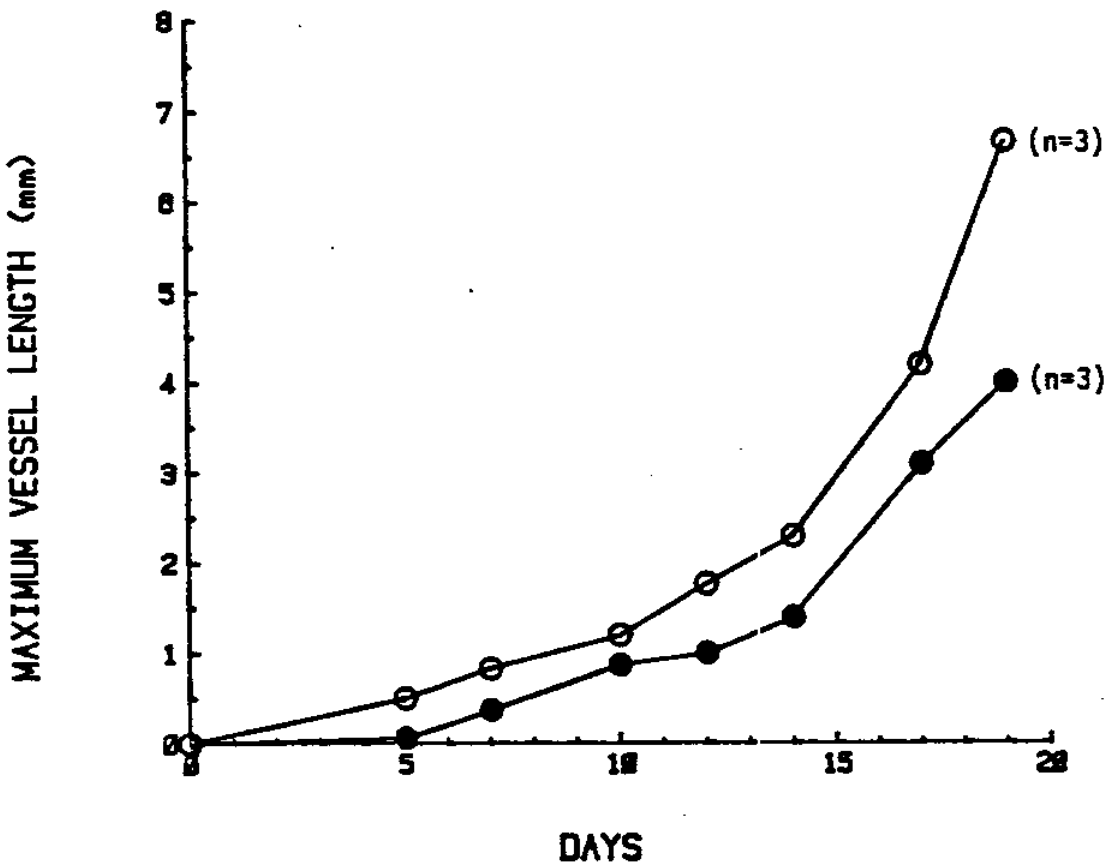

FIGURE 3 : Inhibition of tumor neovascularization by veal scapular cartilage extracted for 1 day in 1M guanidine.

The right cornea was implanted with a polymer pellet containing .11 mg of crude extract by weight (●). The left cornea was implanted with an empty polymer and served as a control (o). Rabbits were sacrificed on day 19, because the tumors in the left eyes became three dimensional and necrotic. The standard deviation of the maximal vessel lengths measured for the three animals tested with veal crude extract polymer pellets or empty pellets were as follows: for veal extract, on days 5,7,10,12,14,19 the standard deviations were .06, .12, .12, 0,.35, 2.5, 3.4 respectively. For the controls, on days 5,7,10,12,14,19 the standard deviations were .17, .32, .26, .76, .75, 1.7, 1.2 respectively.

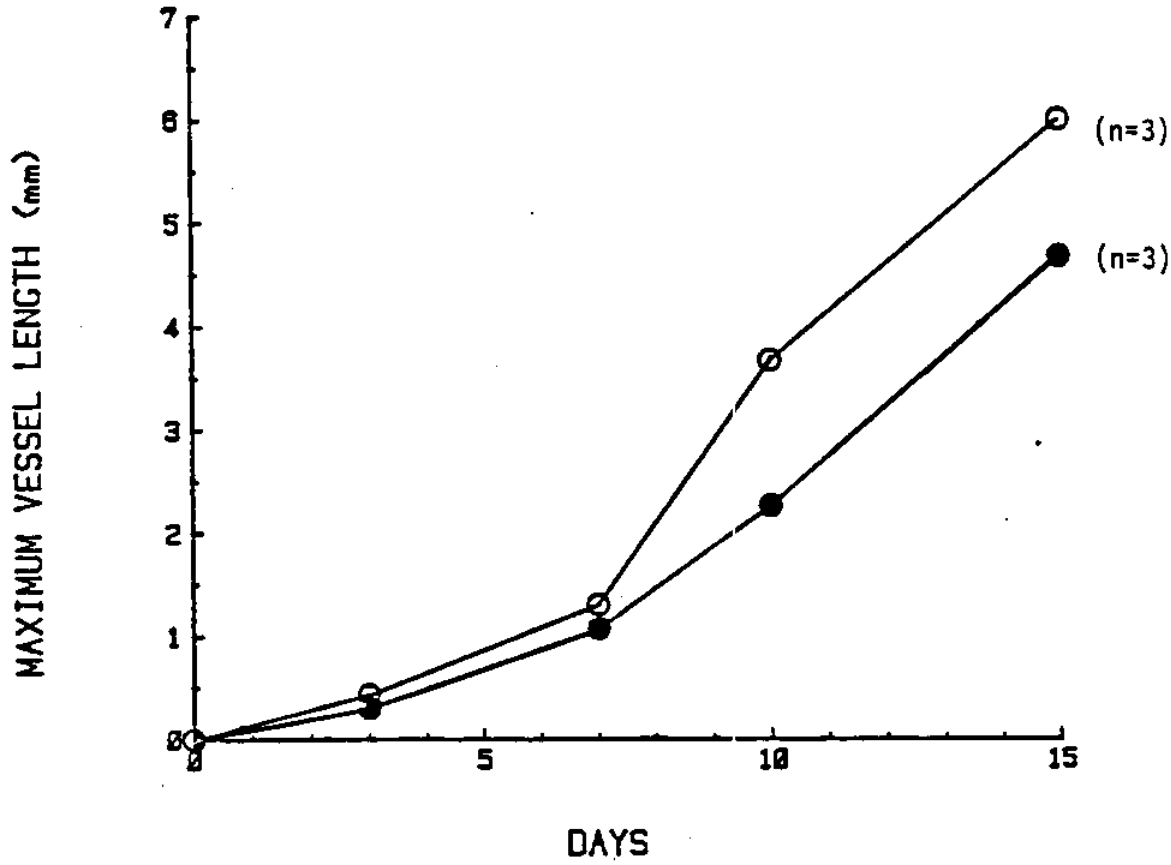

FIGURE 4: Inhibition of tumor neovascularization by the basking shark fin cartilage extracted for 1 day in 1M guanidine.

The right cornea was implanted with a polymer pellet containing .11 mg of crude extract by weight (●). The left cornea was implanted with an empty polymer and served as a control (o). Rabbits were sacrificed on day 15, because the tumors in the left eyes became three dimensional and necrotic. The standard deviation of the maximal vessel lengths measured for the three animals tested with shark crude extract polymer pellets or empty pellets were as follows: for shark extract, on days 3,7,10,15 the standard deviations were .1, .4, .25, .58 respectively. For the controls, on days 3, 7, 10, 15, the standard deviations were .15, .17, 1.6, 1.7 respectively.

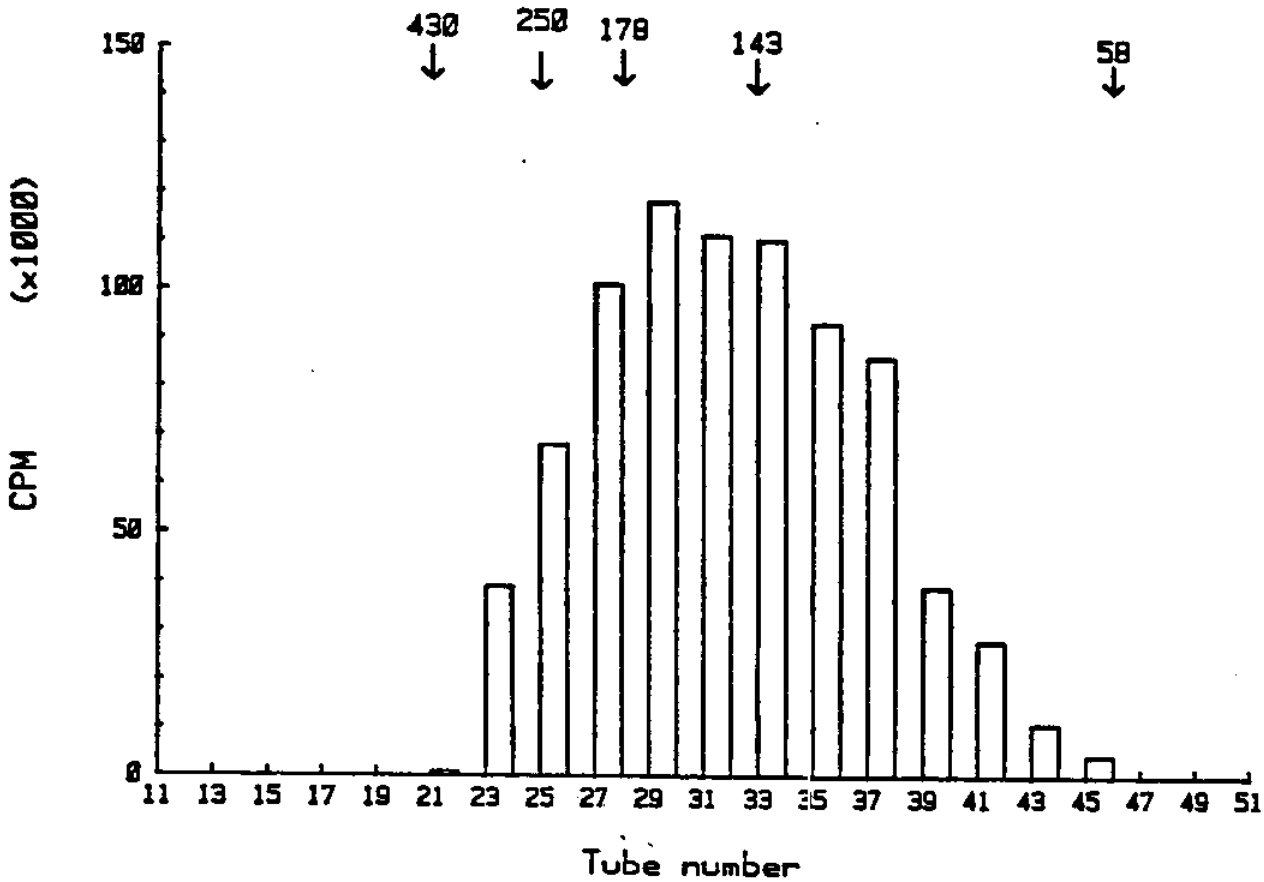

Figure 5: A-0.5M column purification of cellular growth factor extracted from the basking shark fin cartilage.

Cellular growth factor was measured by the incorporation of tritiated thymidine by 3T3 cells. The bars represent growth factor activity in counts per minute.